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Three Distinct Mechanisms Facilitate Genetic Isolation of Sympatric Wheat Streak Mosaic Virus Lineages

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Cross-protection and vector transmission bottlenecks have been proposed as mechanisms facilitating genetic isolation of sympatric viral lineages. Molecular markers were used to monitor establishment and resolution of mixed infections with genetically defined strains of wheat streak mosaic virus (WSMV). Two closely related WSMV strains from the U.S. (Type and Sidney 81) exhibited reciprocal cross-protection in wheat, confirming this classic phenomenon as a mechanism of genetic isolation. In contrast, cross-protection between either U.S. strain and the divergent El Batán 3 strain from Mexico was unilateral, erratic, and only partially effective. Distribution of WSMV strains within individual leaves of plants supporting a mixed infection of Type and Sidney 81 was spatially nonuniform. Strain distribution among individual tillers of coinfected plants also was heterogeneous, with some containing either Type or Sidney 81 alone and some containing both. Transmission by wheat curl mites, acquiring virus from source plants simultaneously infected with both Type and Sidney 81, often resulted in test plants bearing only a single WSMV strain. Spatial subdivision of virus strains within coinfected plants likely contributed to vector transmission bottlenecks during acquisition. Collectively, these three distinct mechanisms enhance genetic isolation of individual viral lineages, and together with stochastic processes, may explain generation and maintenance of genetic diversity in field populations.

INTRODUCTION

Wheat streak mosaic virus (WSMV) is the type species of the newly recognized genus *Tritimovirus* within the family *Potyviridae* (Stenger *et al.,* 1998). The Sidney 81 and Type strains of WSMV share 97.6% nucleotide sequence identity (Choi *et al.,* 2001) and are representative of WSMV genotypes in the U.S. (Chenault *et al.,* 1996). The El Batán 3 strain was recovered from an isolated population of WSMV in Mexico (Sánchez-Sánchez *et al.,* 2001) and shares only ~79% nucleotide sequence identity with Type and Sidney 81 (Choi *et al.,* 2001).

Field isolates of WSMV are variable in symptom severity and physical properties of the capsid protein (CP; McKinney, 1937, 1956; Carroll *et al.,* 1982; Brakke *et al.,* 1990; Montana *et al.,* 1996). This variation is paralleled by moderate levels of CP cistron nucleotide sequence diversity among isolates (Chenault *et al.,* 1996; McNeil *et al.,* 1996). WSMV isolates collected from individual fields exhibited nearly as much sequence diversity as the total diversity among five regions of Nebraska (McNeil *et al.,* 1996). Despite the occurrence of many distinct genotypes in close proximity, only 11 of 472 (~2%) plants sampled were infected with more than one WSMV genotype. It was hypothesized that founder effects imposed by vector transmission bottlenecks and subsequent prevention of superinfection by cross-protection could explain the paucity of mixed infections (McNeil *et al.,* 1996).

The phenomenon of cross-protection, where infection by one (protector) virus prevents subsequent superinfection by a closely related (challenge) virus, has been known for decades (McKinney, 1929). Although the mechanistic basis of cross-protection is not fully understood (Hamilton, 1980; Fraser, 1985; Sherwood, 1987), one outcome may be effective genetic isolation of closely related viral lineages in a field population. Cross-protection has been demonstrated with mild (protector) and severe (challenge) strains of WSMV (McKinney, 1956). However, reliance on symptoms as an indicator of cross-protection restricted which isolates could be evaluated and precluded examination of strain distribution in coinfected plants.

WSMV is transmitted in nature by the wheat curl mite (Slykhuis, 1955), *Aceria tosichella* (Keifer) (Keifer, 1969; Amrine and Stasny, 1994). WSMV is acquired only by

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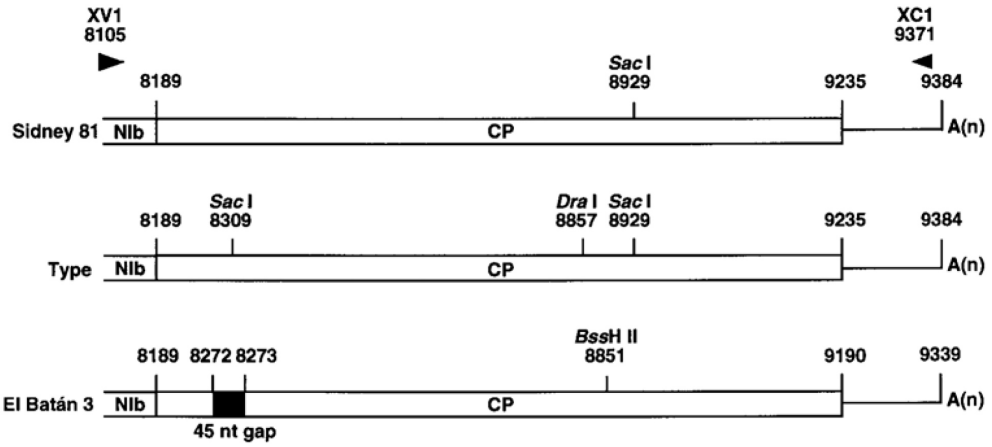

FIG. 1. Physical maps of the coat protein (CP) cistron and flanking regions for wheat streak mosaic virus strains Sidney 81, Type, and El Batán 3. Primer annealing sites (XV1 and XC1), relevant restriction endonuclease cleavage sites, and nucleotide coordinates are indicated. Note that El Batán 3 has a 45 nt gap in the CP cistron relative to the other strains.

nymphs and transmitted by both nymphs and adults; however, the transmission efficiency declines with the age of the adults (Slykhuis, 1955; del Rosario and Sill, 1965). Although vector transmission is widely considered an important bottleneck restricting genetic diversity, data in support of this hypothesis are limited. Alteration of biological properties (Broadbent *et al.,* 1996) or haplotype composition (Ayllón *et al.,* 1999) in citrus tristeza virus, and infrequent establishment of cucumovirus reassortants (Perry and Francki, 1992; Fraile *et al.,* 1997), suggest bottlenecks resulting from aphid transmission. Bottlenecks with rice ragged stunt virus are inferred from the segregation of point mutations in genome segment 9 following propagative transmission by leafhoppers (Suga *et al.,* 1995). Currently, there is no information on transmission by *A. tosichella* serving as a bottleneck.

Closely related viral genotypes may be viewed as operational taxonomic units (OTUs). When OTUs have overlapping geographic distributions, they are sympatric. Coexistence of sympatric OTUs requires separation by reproductive or biotic barriers. Identification of mechanisms isolating viral genotypes into separate lineages is essential for understanding plant virus population dynamics and evolution. In this report, we examined both establishment and resolution of mixed infections using three genotypically defined strains of WSMV. The use of unambiguous molecular markers to distinguish WSMV strains permitted reciprocal cross-protection assays, evaluation of the spatial distribution of strains in tissues of coinfected plants, and strain composition in test plants following vector transmission from source plants bearing mixed infections. These experiments indicate that cross-protection, vector transmission bottlenecks, and subdivided populations within a plant promote genetic isolation of viral lineages and likely contribute to establishment and maintenance of complex field populations.

RESULTS

Establishment of mixed infections and cross-protection between WSMV strains

Genotypic differences within the CP cistron (Fig. 1) allowed discrimination of WSMV strains present in single or mixed infections. Digestion of reverse transcriptase-polymerase chain reaction (RT-PCR) products with *Sac*I produced strain-specific electrophoretic patterns (Fig. 2). To eliminate the possibility of partial endonuclease digestion confounding the results, strain composition was verified by *Dra*I or *Bss*H II digestion, as appropriate for the mixture of virus stains inoculated. Control experiments using purified plasmids as templates showed that a strain could be detected in the presence of a 10-fold (but not 50-fold) excess of another (data not shown).

Simultaneous inoculation of wheat (*Triticum aestivum* L., cv. Centurk) seedlings with pairs of WSMV strains yielded primarily mixed infections (Fig. 2, Table 1). Prior infection of plants by either Type or Sidney 81 protected against superinfection (80–100% effective) upon chal-

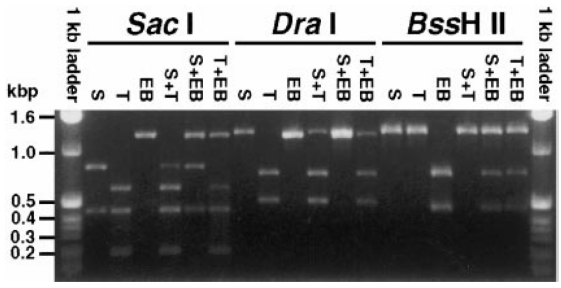

FIG. 2. Determination of strain composition within primary shoots of wheat plants individually infected or coinfected with wheat streak mosaic virus strains Sidney 81 (S), Type (T), and El Batán 3 (EB). Presented are restriction endonuclease digestions (*Sac*I, *Dra*I, *Bss*H II) of RT-PCR products of the coat protein cistron. The sizes in kilobase pairs (kbp) of 1-kb ladder DNA standards are indicated at left.

TABLE 1

Establishment of Mixed Infections and Cross-Protection among Strains of Wheat Streak Mosaic Virus

First inoculum	Second inoculum	Inoculation interval	Strain (1^{st}:2^{nd})[a] Experiment 1	Strain (1^{st}:2^{nd})[a] Experiment 2
Sidney 81	—	—	10:—	10:—
Type	—	—	10:—	10:—
El Batán 3	—	—	10:—	10:—
Sidney 81	Type	Simultaneous	10:10	10:10
Sidney 81	El Batán 3	Simultaneous	10:10	10:10
Type	El Batán 3	Simultaneous	10:10	10:8
Sidney 81	Type	7 days	10:1	10:2[b]
Sidney 81	El Batán 3	7 days	10:0	10:6[b]
Type	Sidney 81	7 days	10:0	10:0
Type	El Batán 3	7 days	10:3[b]	10:9[b]
El Batán 3	Sidney 81	7 days	10:9	10:10
El Batán 3	Type	7 days	10:10	9:10
El Batán 3	Sidney 81	14 days	ND[c]	10:10
El Batán 3	Type	14 days	ND[c]	10:10

[a] Number of plants infected (of 10 inoculated) with the first strain indicated before the colon; number of plants also infected with the second strain indicated after the colon.

[b] Titre of second strain less than first strain when present.

[c] ND = not determined.

lenge inoculation with the other strain (Table 1). In contrast, erratic results were obtained when either U.S. strain was used as the protector to exclude El Batán 3. Effectiveness of cross-protection against El Batán 3 ranged between 10 and 70% (Type) and 60 and 100% (Sidney 81), although successful superinfection generally resulted in lower titers of El Batán 3 relative to that observed in single or simultaneous inoculations (Table 1). However, El Batán 3 did not cross-protect against either U.S. strain, even when challenge inoculations were postponed to 14 days following inoculation with El Batán 3 as the protector.

In planta distribution of WSMV strains in mixed infections

Although bulk samples (comprising several systemically infected leaves) from primary shoots of coinfected plants often contained approximately equal titers of each strain (Fig. 2), this does not demonstrate uniform distribution of strains within plant tissues. To examine the distribution of Type and Sidney 81 in coinfected plants, multiple 1-mm disk samples from individual systemically infected leaves were assayed. Disk samples derived from the same leaf varied in both strain composition and relative titers, indicating that strain distributions were not uniform (Fig. 3A, Table 2). Within individual coinfected leaves, some disk samples contained Type only, while others contained Sidney 81 only. The remaining disk samples contained both strains in varying amounts.

Spatial partitioning was further examined through sampling individual tillers produced by coinfected plants.

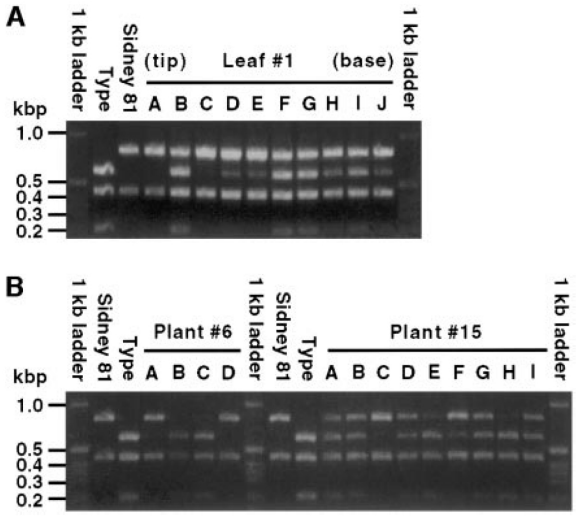

FIG. 3. Nonuniform distribution of wheat streak mosaic virus strains Type and Sidney 81 within coinfected wheat plants. Presented are *Sac*I digests of RT-PCR products of the coat protein cistron amplified from (A) discrete 1-mm disks (samples A–J) from the same systemically infected leaf (#1) or from (B) separate tillers of two plants (#6, samples A–D; #15, samples A–I) produced after systemic infection of the primary shoot. *Sac*I digestions of RT-PCR products from plants singly infected with either Type or Sidney 81 are shown for comparison. The sizes in kilobase pairs (kbp) of 1-kb ladder DNA standards are indicated at the extreme left.

Plants known to be infected with both Type and Sidney 81 in the primary shoot were reexamined after tiller development. Bulk samples from individual tillers present 7 weeks postinoculation were assayed for virus strain composition (Fig. 3B, Table 3). As with samples derived from the same leaf, the distribution of virus strains in tillers of a coinfected plant was not uniform. Some (12%) tillers contained only one strain, while other tillers from the same plant contained both strains (Fig. 3B, Table 3). In tillers containing both strains, relative titer varied and did not appear biased toward one strain. However, no segregation was observed in individual

TABLE 2

Distribution and Relative Titres[a] of Wheat Streak Mosaic Virus Type (T) and Sidney 81 (S) Strains within Coinfected Wheat Leaves

Leaf[b]	*n*[c]	S only	T only	S = T	S > T	T > S
1	10	2	0	5	3	0
2	12	0	5	6	0	1
3	12	0	3	8	0	1
4	12	1	8	1	0	2
5	9	0	0	3	1	5
Totals	55	3	16	23	4	9

[a] Based on strain-specific fragments produced by endonuclease digestion of RT-PCR products.

[b] Each leaf sampled was systemically infected and from a separate plant.

[c] Number of 1-mm disks sampled from a single leaf.

TABLE 3

Distribution and Relative Titres[a] of Wheat Streak Mosaic Virus Type (T) and Sidney 81 (S) Strains in Tillers of Coinfected Wheat Plants

Plant	Primary shoot	Tillers					
		n^b	S only	T only	S = T	S > T	T > S
1	S = T	7	2	0	2	2	1
2	S = T	7	0	0	2	3	2
3	S = T	4	0	0	1	2	1
4	S = T	3	0	0	2	0	1
5	S = T	9	1	0	4	1	3
6	S > T	4	1	1	0	1	1
7	S = T	11	0	0	9	0	2
8	S = T	4	0	0	1	0	3
9	S = T	9	1	2	5	1	0
10	S = T	8	0	0	4	1	3
11	S = T	8	1	0	3	2	2
12	S = T	9	1	0	6	2	0
13	S = T	9	0	0	3	2	4
14	S = T	5	0	1	2	0	2
15	S = T	9	1	0	5	1	2
16	S = T	2	0	1	1	0	0
Totals		108	8	5	50	18	27

[a] Based on strain-specific fragments produced by endonuclease digestion of RT-PCR products.
[b] Number of tillers examined per plant.

tillers from plants coinfected with Sidney 81 and brome mosaic virus (BMV). Both viruses occurred simultaneously in every tiller of every plant tested (Table 4).

Resolution of mixed infections by vector transmission

Transmission efficiency by individual wheat curl mites given access to source plants, coinfected with Type and Sidney 81, was 18.5% (Table 5). Although the percentage of mites transmitting virus to test plants varied (10–40%) depending upon the source plant, each strain was detected in at least one test plant for five out of seven source plants. No virus transmission events were observed for mites given access to uninfected wheat plants. Of 23 transmission events by individual mites, 20 (87%) infected test plants contained only Type (30%) or only Sidney 81 (57%). Only three (13%) infected test plants were dually infected with both Type and Sidney 81.

TABLE 4

Distribution of Wheat Streak Mosaic Virus Strain Sidney 81 (S) and Brome Mosaic Virus (BMV) in Tillers of Coinfected Wheat Plants

Plant	Number of tillers	S only	BMV only	S + BMV
1	5	0	0	5
2	8	0	0	8
3	4	0	0	4
4	3	0	0	3
5	3	0	0	3
6	4	0	0	4
7	4	0	0	4
8	6	0	0	6
9	6	0	0	6
10	6	0	0	6
11	6	0	0	6
12	5	0	0	5
13	5	0	0	5
14	3	0	0	3
Totals	68	0	0	68

TABLE 5

Transmission of Wheat Streak Mosaic Virus Strains Type (T) and Sidney 81 (S) by Individual Wheat Curl Mites Given Access to Coinfected Wheat Plants[a]

Source plant	Test plants				
	n^b	Number positive	S	T	S + T
Uninfected[c]	16	0 (0%)	0	0	0
1	20	2 (10%)	0	2	0
2	20	3 (15%)	2	1	0
3	20	2 (10%)	2	0	0
4	5	2 (40%)	1	0	1
5	19	7 (37%)	4	2	1
6	20	4 (20%)	3	0	1
7	20	3 (15%)	1	2	0
Totals	124	23 (18.5%)	13	7	3

[a] All virus infected plants used as acquisition sources contained similar titres of both strains in the primary shoot.
[b] Number of test plants per source plant.
[c] Source plant was not inoculated with WSMV strains.

DISCUSSION

Mechanisms of genetic isolation among sympatric viral lineages

Temporal separation of inoculation by only a few days is sufficient for the first virus to prevent superinfection by a closely related virus. Although 7 days was used as the standard interval between protector and challenge inoculations, this is a relatively brief period given the length of a typical growing season. Furthermore, other studies suggest that cross-protection may operate with only 48 h between inoculations, as demonstrated with marked genotypes of the potyvirus zucchini yellow mosaic virus (Desbiez *et al.,* 1997). Therefore, a plant is susceptible to multiple infection by closely related viral lineages only during a brief window of time. Cross-protection between either U.S. strain and the more divergent El Batán 3 strain was less predictable. This is consistent with previous findings that show that the extent of cross-protection correlates with degree of genetic relatedness between protecting and challenging virus strains (Fraser, 1985; Ponz and Bruening, 1986).

Mixed infections were the usual outcome of simulta-

neous inoculation under laboratory conditions. Nevertheless, virus strain mixtures became spatially discontinuous in systemically infected tissues. This occurred both in small regions within leaves of primary shoots and in entire secondary shoots (tillers). Since most plant viruses do not invade meristematic cells, invasion of newly developed tissues occurs after formation of vascular connections with older tissues. The implication is that virus movement through these newly made connections is a highly restrictive process. Once an infection focus is established by one lineage, spatial autonomy is maintained by cross-protection at the cellular level, precluding invasion by closely related lineages. No such interference occurs between unrelated viruses, however. Predictably then, spatial segregation of distinct virus species (WSMV and BMV) was never observed in mixed infections.

Because Type and Sidney 81 produce similar symptoms in wheat, nonuniform distribution is not visually discernible. Strains with distinctive symptoms, however, should show patchy distributions. In fact, this is how some strains and mutants were originally noticed and isolated; the first being yellow sector mutants of tobacco mosaic virus studied by McKinney (1929, 1935). Another early example is the red necrotic lesion mutant of tobacco necrosis virus that originates as sectors within white lesions (Fulton, 1952). Our data are consistent with these observations and indicate that movement and distribution of virus within a plant are spatially constrained, unlike the situation in liquid-cell cultures of animal and bacterial viruses. Thus, plant anatomy and development impose profound restrictions on virus population growth and evolutionary dynamics that are not accounted for by models based on phage or animal viruses cultured in well-mixed flasks.

Wheat curl mite transmission clearly acted as a bottleneck resolving mixed infections. Because Type and Sidney 81 are each readily transmitted by mites as pure cultures (Brakke, 1971; Choi *et al.,* 1999), it is unlikely that our results could be explained by differences in transmission efficiency intrinsic to the two strains. Bottlenecking may occur during one or more phases of transmission. Acquisition bottlenecks could result from spatial segregation of virus strains concomitant with limited movement of immature (virus acquiring) mites. Postacquisition bottlenecks are also possible during the inoculation phase, particularly if infection is established by a limited number of virions delivered to host cells through the feeding activity of the vector.

Consequences of genetic isolation among sympatric viral lineages

Cross-protection minimizes the occurrence of mixed infections. Nonuniform spatial distribution in mixed infections reduces the number of cells where different viral lineages occur together. Vector transmission bottlenecks further tend to resolve mixed infections. Collectively, these three mechanisms promote genetic isolation of sympatric viral lineages, providing a plausible explanation for the rarity of mixed WSMV infections in the field.

Our results demonstrate spatial segregation of viral lineages within a plant, but do not exclude mixed infections in some cells. Both strains were detected in more than half of the 1-mm disk samples from systemically infected leaves of plants simultaneously inoculated with two strains. These either represent regions of doubly infected cells and/or overlapping sectors of predominately singly infected cells. In either case, there should be some cells where different lineages arrive at nearly the same time. The resulting doubly infected cells provide opportunities for recombination. Thus, spatially partitioned genotypes are able to retain their identities as distinct lineages, yet may occasionally exchange genetic information to repair genetic lesions or capture beneficial mutations from other lineages. It would be of interest, therefore, to examine virus distribution in coinfected plants using techniques whereby the presence or absence of different genotypes within individual cells can be ascertained.

WSMV populations within a plant are clearly structured. There are sectors dominated by single lineages that have restricted contact with others. This has important implications for virus population genetics that apply not only to preexisting strains, but also to new variants arising by mutation. Subdivision of viral lineages within a plant, with concomitant bottlenecks during systemic movement and vector transmission, will reduce the effective population size. The contribution of genetic drift to evolution is enhanced by small effective population size compared to a population with a large effective population size (Nei, 1987), such that mutations will become fixed in a lineage more rapidly. An examination of the Type, Sidney 81, and El Batán 3 genomes indicate that although much of the genome is conserved and appears subject to negative selection, most of the variation among these WSMV strains may be explained by stochastic processes such as genetic drift (Choi *et al.,* 2001). As the three mechanisms of genetic isolation described here are conducive to genetic drift, they may significantly effect both diversity and divergence within WSMV.

MATERIALS AND METHODS

Virus strains and inoculations

The Sidney 81 strain (GenBank accession no. AF057533) was recovered near Sidney, Nebraska in 1981 (Brakke *et al.,* 1990). The Type strain (GenBank accession no. AF285169) was collected in Kansas in 1932 (McKinney, 1937). The El Batán 3 strain (GenBank acces-

sion no. AF285170) originated in the Central Highlands of Mexico in 1996 (Sánchez-Sánchez *et al.,* 2001).

Frozen leaf tissue infected with each WSMV strain was macerated in water (1:10 w:v) and mechanically inoculated onto 7- to 10-day-old wheat seedlings. In cross-protection assays, pairs of WSMV strains were sequentially inoculated to wheat seedlings with challenge inoculations delayed for 7 or 14 days following protector inoculations. Pairs of WSMV strains also were inoculated to wheat seedlings simultaneously as equal mixtures. Bulk samples (several leaves) of systemically infected tissues of the primary shoot were harvested 21 days after challenge inoculation.

Plants demonstrated to contain a mixed infection of Type and Sidney 81 in primary shoots were maintained an additional 4 weeks to allow tiller formation. Mixed infections of Sidney 81 and BMV also were established and examined for virus distribution in tillers. In both cases, samples from individual tillers consisted of several leaves. Strain distribution within individual leaves of plants containing mixed infections of Type and Sidney 81 was assessed by examining small (~1 mm) disks of tissue taken 3 cm apart on alternating sides of the midvein. All plant samples were stored at −20°C prior to extraction.

Molecular markers for virus strain identification

Total nucleic acids were extracted from frozen tissue samples and viral RNA was reverse transcribed as previously described (McNeil *et al.,* 1996). PCR primers were designed to anneal to perfectly conserved regions flanking the CP cistron (Fig. 1). The sequence of the upstream primer XV1 (5′-*GATCC*GTTGAGGATTTGTACTT-3′) corresponds to nucleotides 8105 to 8121 of Sidney 81, Type, and El Batán 3. Five unmatched bases (italics) at the 5′-end of primer XV1 were included to achieve an annealing temperature similar to that of primer XC1 after the first cycle of PCR. The downstream primer XC1 (5′-AACCCACACATAGCTACCAAG-3′) is complementary to nucleotides 9371–9351 of Type and Sidney 81, and nucleotides 9326–9306 of El Batán 3. PCR was performed for 30 cycles using *Taq* DNA polymerase and amplified a 1267-bp product of Sidney 81 or Type. The El Batán 3 PCR product was 45 bp smaller in length due to a gap in the El Batán 3 sequence located near the 5′-end of the CP cistron (Choi *et al.,* 2001; Fig. 1). Full-length BMV RNA 3 was detected by RT-PCR using the primers BMV-3′ (5′-GATCCGCGGTCTCTTTTAGAGATTTAC-3′) and BMV-5′ (5′-AATTAAGCTTACGTAAAATACCAACTAATTC-3′). Restriction endonucleases were identified that yielded diagnostic fragments upon digestion of PCR products for each of the three strains (Fig. 1). Specifically, *Bss*H II cuts the El Batán 3 PCR product once and the others not at all. *Sac*I cuts Sidney 81 once, Type twice, and does not cut El Batán 3. *Dra*I cuts Type once while not cutting the others. Restriction endonuclease products were separated by electrophoresis in 1.2% agarose gels and visualized by staining with ethidium bromide.

Vector transmission assays

Seven wheat plants verified by RT-PCR as coinfected with Type and Sidney 81 served as source plants. Aviruliferous wheat curl mites reared in cages on uninfected wheat plants were transferred in groups to caged source plants for a 14-day acquisition access period. Another group of aviruliferous wheat curl mites was placed on caged uninfected wheat plants as a control. Following the acquisition access period, individual mites (one per test plant) were placed onto 7-day-old test plants for an inoculation access period of 21 days. Following the inoculation access period, bulk samples of the primary shoot of each test plant were collected and stored at −20°C until extracted. Nucleic acid extraction and molecular marker analysis were as described above to determine strain composition in infected test plants.

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